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ANTIBODIES TO MULTIPLE TICK-BORNE PATHOGENS OF BABESIOSIS, EHRLICHIOSIS, AND LYME BORRELIOSIS IN WHITE-FOOTED MICE

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ABSTRACT: Serum samples from *Peromyscus leucopus* (white-footed mouse), collected in Connecticut (USA) in 1983, 1985, and during 1990 to 1993, were analyzed by an enzyme-linked immunosorbent assay (ELISA) or indirect fluorescent antibody (IFA) staining methods for antibodies to *Borrelia burgdorferi* (strain 2591), *Babesia microti*, *Ehrlichia chaffeensis* (Arkansas strain), and *Ehrlichia equi* (MRK strain). Of the 294 serum samples tested, 160 (54%) contained immunoglobulins to one or more of these pathogens. There were antibodies to two or more etiologic agents in 77 (48%) of the seropositive mice. Although it was uncommon to detect coexisting antibodies to all four pathogens (n = 5 positive mice), *E. chaffeensis*-reactive antibodies or immunoglobulins to *E. equi* were present along with those produced to *B. burgdorferi* and *B. microti* in 24 other mice. These rodents carry antibodies to several tick-borne pathogens at numerous sites in Connecticut and may play a role in the epizootiology of ehrlichiosis as well as babesiosis and Lyme borreliosis.

Key words: Babesiosis, ehrlichiosis, Lyme borreliosis, *Peromyscus leucopus*, white-footed mice, *Borrelia burgdorferi*, *Babesia microti*, *Ehrlichia chaffeensis*, *Ehrlichia equi*.

INTRODUCTION

White-footed mice (*Peromyscus leucopus*) are chief reservoirs for *Babesia microti* and *Borrelia burgdorferi* (J.F. Anderson et al., 1986, 1991), the etiologic agents of human babesiosis and Lyme borreliosis, respectively. Both diseases occur in or near forested areas of the northeastern and upper midwestern United States (J.F. Anderson et al., 1991; Krause et al., 1991; Herwaldt et al., 1995) where *Ixodes scapularis* ticks, white-footed mice, and white-tailed deer (*Odocoileus virginianus*) are abundant. Infections with a *Babesia* sp.-like organism also have been reported in northern California (USA) (Persing et al., 1995) where *Ixodes pacificus* occurs. Larvae and nymphs of *I. scapularis* can acquire one or both pathogens when they feed from infected mice. Transmission of infectious agents to vertebrate hosts occurs most frequently when infected nymphs feed (Piesman et al., 1987).

Human cases of babesiosis and Lyme borreliosis are well documented in the northeastern United States (Cartter et al., 1989; Gadbaw et al., 1989; Meldrum et al., 1992), but human granulocytic ehrlichiosis (HGE) also occurs there (Hardalo et al., 1995) and in the upper Midwest (Bakken et al., 1994; Chen et al., 1994). Based on clinical and laboratory findings, HGE infections can be mild or severe. Human monocytic ehrlichiosis (HME) is far more prevalent in the southern United States (B. E. Anderson et al., 1991, 1993) but may be present in northern states as well. On analyses of human sera, coexisting antibodies to *B. microti*, *Ehrlichia chaffeensis* (agent of HME) or *Ehrlichia equi* (suspected agent of HGE) were found in some persons who were diagnosed with Lyme borreliosis and who had antibodies to *B. burgdorferi* (Magnarelli et al., 1995a). Moreover, in polymerase chain reaction (PCR) analyses of *Ixodes scapularis* ticks collected from sites in southern Connecticut (USA), the DNA of the HGE agent was detected in 59 (50%) of 118 adult and in one of two nymphs tested (Magnarelli et al., 1995b). Substantially lower prevalences of infection (10% and 15%) were

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reported for *I. scapularis* in Wisconsin (USA) by Pancholi et al. (1995) and in Nantucket, Massachusetts (USA) by Telford et al. (1996). In laboratory studies (Telford et al., 1996), larval ticks of this species acquired infection by feeding on mice that carried a human-derived strain of the HGE agent. Nymphal ticks then transmitted the bacterium to other mice. Patients in Wisconsin and Minnesota (USA), who were diagnosed as having HGE, recalled being bitten by *I. scapularis* (Chen et al., 1994). Accordingly, this tick species is probably an important vector of *E. equi* or a closely related organism. Since larvae and nymphs of *I. scapularis* frequently parasitize white-footed mice, these rodents may be infected with ehrlichiae and serve as reservoirs of infection. This study was conducted to determine if white-footed mice contain antibodies to *E. chaffeensis* or *E. equi* and to assess prevalence of mouse exposure to multiple tick-borne pathogens in nature.

MATERIALS AND METHODS

White-footed mouse serum samples were selected from stored collections kept at −60 C at the Connecticut Agricultural Experiment Station, New Haven, Connecticut. Sera were from animals captured in Sherman box traps (H.B. Sherman Traps, Inc., Tallahassee, Florida USA) from January through November of 1983, 1985, and during the period 1990 to 1993 in Connecticut. Some serum samples chosen for analyses of antibodies to *E. chaffeensis* and *E. equi* had been screened earlier and contained antibodies to *B. burgdorferi*, *B. microti*, or both (J.F. Anderson et al., 1991; Magnarelli et al., 1994). Mice were from forested areas of 22 towns, most of which are located between Stonington (41°28′N, 71°55′W) and Stamford (41°8′N, 73°33′W) in southern Connecticut where *I. scapularis* ticks are abundant and numerous human cases of Lyme borreliosis have been reported (Cartter et al., 1989). Most serum samples in the present study were from the following towns: Chester, East Haddam, Killingworth, Lyme, Madison, Mystic, North Stonington, Old Lyme, Stamford, Stonington, and Waterford. In addition to serum samples from field-collected animals, sera were obtained from 13 laboratory-born white-footed mice and were used as negative controls in antibody analyses.

Serum samples were available from 20 white-footed mice that had been included in isolation studies for *B. burgdorferi* and *B. microti* (J.F. Anderson et al., 1991). To culture *B. burgdorferi*, tissues of mouse bladders, kidneys, spleens, and (or) ears were introduced into duplicate tubes of Barbour-Stoenner-Kelly medium with or without 0.1% agarose as described by J.F. Anderson et al. (1986, 1991). Spirochetes in culture tubes were identified by using indirect fluorescent antibody (IFA) staining methods with monoclonal antibody H5332, which is specific for outer surface protein A of *B. burgdorferi*. This protein is common to North American isolates of *B. burgdorferi sensu stricto*. Isolation of *B. microti* was accomplished by inoculating blood from each mouse into Golden Syrian hamsters (*Mesocricetus auratus*). Details on inoculation procedures, fixation and staining of infected erythrocytes on glass microscope slides, and microscopic examinations for *B. microti* have been reported (J.F. Anderson et al., 1991).

An enzyme-linked immunosorbent assay (ELISA) (Magnarelli et al., 1994) was used to quantitate antibody concentrations to *B. burgdorferi* (strain 2591). This assay was designed to detect total serum immunoglobulins and included an affinity-purified peroxidase-labeled goat anti-*P. leucopus* immunoglobulin (H- and L-chain specific), prepared by Kirkegaard and Perry Laboratories (Gaithersburg, Maryland, USA) diluted to 1:2000 in phosphate buffer saline (PBS) solution.

Antibodies to *B. microti* were detected by using IFA staining procedures. The antigen consisted of infected erythrocytes obtained from Golden Syrian hamsters that had been inoculated with whole blood from an infected person who lived in Stonington, Connecticut, and who was diagnosed with human babesiosis (J.F. Anderson et al., 1991). Materials and methods used to prepare this antigen for testing and for washing slides have been described by J.F. Anderson et al. (1991). In short, serial twofold dilutions of mouse sera were analyzed for total antibodies with a 1:40 dilution of polyvalent fluorescein-conjugated goat anti-mouse immunoglobulins (Organon Teknika Corp., Durham, North Carolina, USA) mixed with 1.0% Evan's Blue. In analyses of 13 serum samples from laboratory-born individuals unexposed to *B. microti*, there were no false positive reactions when a serum dilution of 1:80 was tested. Therefore, distinct fluorescence of *B. microti* at or above this cut-off dilution was considered positive. Two positive control serum samples, having homologous antibody titers of 1:1,280 and 1:5,120 to *B. microti*, originated

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from two white-footed mice found to be harboring this pathogen in isolation studies.

In IFA staining methods used to detect ehrlichial antibodies, antigens of *E. chaffeensis* (Arkansas strain) in DH82 cells or *E. equi* (MRK strain) in horse neutrophils were fixed to glass microscope slides. The sources of these reagents have been reported by Magnarelli et al. (1995b). Fluorescein-conjugated antibodies, used in tests with *B. microti*, were suitable in analyses with ehrlichial antigens as the second antibody. This conjugate, diluted to 1:40 (without Evan's Blue) in PBS solution (pH = 7.2), allowed for homologous reactivity of antibodies and antigen without excessive background fluorescence. Another fluorescein isothiocyanate-labeled goat anti-*P. leucopus* antibody reagent (H- and L- chain specific) was purchased from Kirkegaard and Perry Laboratories, and used at a dilution of 1:20 in comparative tests. When 13 sera from laboratory-born *P. leucopus*, which were not exposed to ehrlichiae, were screened against *E. chaffeensis* or *E. equi* using either conjugate, there were no false positives at a serum dilution of 1:80. Weak non-specific reactions were observed for four sera when serum dilutions of 1:10 and 1:20 were tested. There was one weak false positive reaction when a serum dilution of 1:40 was tested. Accordingly, distinct fluorescence of ehrlichiae at or above a serum dilution of 1:80 was considered positive. As in studies with *B. microti*, grading the intensity of FA reactions to determine titration end points was done conservatively. Positive control sera from white-footed mice known to be infected with *E. chaffeensis* or *E. equi*, as determined by PCR or culture methods, were unavailable. However, reactivity of ehrlichial antigens was verified by testing human serum samples from patients diagnosed with HME or HGE (Magnarelli et al., 1995b). Moreover, an effort was made to test mouse sera that had been obtained from animals caught in East Haddam and Lyme, Connecticut, areas where the DNA of the HGE agent was found in *I. scapularis* ticks (Magnarelli et al., 1995b).

RESULTS

Serum samples collected from white-footed mice contained antibodies to *E. chaffeensis*, *E. equi*, or both. Of the 294 serum specimens tested, 31 (11%) were considered positive in each assay (Table 1). Seven serum samples contained antibodies to both antigens. When 26 or more sera were tested, seroprevalence ranged from 7% to 21% for *E. chaffeensis* and from 4% to 36% for *E. equi*. In general, the numbers of positive serum samples recorded during any given year of sampling were relatively low. However, positive serum samples were collected in the 11 following towns: Chester, Cobalt, East Haddam, Fairfield, Lyme, Madison, Montville, Mystic, Old Lyme, Stonington, and Waterford. Of the 119 mouse sera tested from the towns of East Haddam and Lyme, 29 (24%) contained antibodies to *E. chaffeensis*, *E. equi*, or both.

TABLE 1. Detection of serum antibodies to *Ehrlichia chaffeensis* and *Ehrlichia equi* by indirect fluorescent antibody staining methods in white-footed mice collected in Connecticut during 1983, 1985, and 1990 to 1993.

Sampling year	Number of serum samples tested	Number (%) with antibodies[a] to	
		E. chaffeensis	*E. equi*
1983	26	3 (12)	1 (4)
1985	65	6 (9)	3 (5)
1990	31	2 (7)	11 (36)
1991	2	0	0
1992	28	6 (21)	1 (4)
1993	142	14 (10)	15 (11)
Total	294	31 (11)	31 (11)

[a] A titer of 1:80 or greater was considered positive.

Test results were available for 20 white-footed mice from which *B. burgdorferi*, *B. microti*, or both were isolated. Six mice were infected with either *B. burgdorferi* (n = 3) or *B. microti* (n = 3), while the remaining 14 mice harbored both pathogens. The three animals infected only with *B. burgdorferi* carried antibodies to this agent and *B. microti* but lacked serologic evidence of exposure to ehrlichiae. In the three mice infected with *B. microti* and not *B. burgdorferi*, two had antibodies to *B. microti*, one contained antibodies to *B. burgdorferi*, and one had immunoglobulins to *E. equi*. Of the 14 mice infected with both *B. burgdorferi* and *B. microti*, all animals developed homologous antibodies to both pathogens. Two of these rodents also contained antibodies to *E. chaffeensis*, while nine animals had immunoglobulins to *E. equi*. The 13 serum sam-

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TABLE 2. Reactivity of white-footed mouse sera to *Borrelia burgdorferi*, *Babesia microti*, *Ehrlichia chaffeensis*, and *Ehrlichia equi* by indirect fluorescent antibody staining methods.

Antibodies detected[a] to	Number (%) positive serum samples
B. burgdorferi only	58 (20)
B. burgdorferi and *B. microti*	36 (12)
B. burgdorferi, *B. microti*, and *E. chaffeensis*	7 (2)
all four antigens	5 (2)
B. burgdorferi, *B. microti*, and *E. equi*	17 (6)
B. burgdorferi, *E. equi*, and *E. chaffeensis*	1 (0.3)
B. burgdorferi and *E. equi*	3 (1)
B. burgdorferi and *E. chaffeensis*	5 (2)
B. microti only	11 (4)
B. microti and *E. equi*	2 (0.7)
E. equi only	2 (0.7)
E. chaffeensis only	12 (4)
E. equi and *E. chaffeensis*	1 (0.3)

[a] Note that 134 (46%) of the total 294 serum samples tested had no antibodies to any of the four antigens included in analyses.

TABLE 3. Reactivity of white-footed mouse serum samples to *Babesia microti*, *Ehrlichia chaffeensis*, or *Ehrlichia equi* and frequency distributions of antibody titers for seropositive specimens.

Reciprocal antibody titers[a]	Number of positive serum samples to		
	B. microti	*E. chaffeensis*	*E. equi*
80–160	25	30	15
320–640	30	1	14
1,280–2,560	20	0	1
5,120–10,240	3	0	1

[a] Determined by using indirect fluorescent antibody staining methods.

ples obtained from laboratory-born white-footed mice were negative in all antibody tests.

On the basis of antibody test results, 160 (54%) of 294 mice had been exposed to one or more tick-borne pathogens. Of the positive serum samples, 77 (48%) had antibodies to two or more infectious agents (Table 2). Although it was uncommon to have immunoglobulins to all four pathogens (n = 5 positive sera) present, antibodies to *E. chaffeensis* or *E. equi* coexisted with immunoglobulins produced to *B. burgdorferi* and *B. microti* in 24 other serum samples. There were 17 serum samples that had antibodies to *B. burgdorferi*, *B. microti*, and *E. equi* and seven specimens positive to *B. burgdorferi*, *B. microti*, and *E. chaffeensis*. Seropositives to *B. burgdorferi* only (20% of 294) or to *B. burgdorferi* and *B. microti* (12%) were most prevalent. Conversely, serologic reactivity to *E. chaffeensis*, *E. equi*, or both without immunologic evidence of exposure to *B. burgdorferi* or *B. microti* was less frequent (5%).

Antibody titers to the panel of tick-borne pathogens were highly variable. On comparison of results by IFA staining methods, most antibody titers ranged between 1:80 and 1:640 (Table 3). Of the 78 serum samples containing antibodies to *B. microti*, titration end points for 55 (71%) positive samples were in this range. The remaining sera had titers of 1:1,280 to 1:2,560 (n = 20) or of 1:5,120 to 1:10,240 (n = 3). In similar analyses for ehrlichial antibodies, 60 of 62 titration end points ranged between 1:80 and 1:640. The highest concentrations of antibodies to *E. equi* (1:1,280 and 1:5,120) were recorded for the remaining two sera obtained from mice captured in East Haddam (September 1993) and Mystic (August 1990), respectively. Both of these rodents also had antibodies to *B. burgdorferi* and *B. microti* in similar concentrations. In ELISA results for *B. burgdorferi* antibodies, 90 (68%) of 132 positive sera had titers of 1:1,280 or greater.

Parallel tests were conducted to determine if two commercially available fluorescein-labeled antibody reagents were both suitable for use in IFA staining methods. Ten serum samples were selected for analyses. Five specimens had *E. chaffeensis*-reactive antibodies and five samples had immunoglobulins to *E. equi*. Antibody titers ranged from 1:80 to 1:640 for both sets of sera. By preliminary screening, there were no coexisting antibodies to these ehrlichiae in these samples. In par-

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allel tests with the two conjugates, there were two-fold differences noted in antibody titers for seven serum samples and four-fold changes in titers recorded for three samples. When 13 serum samples from laboratory-born mice were screened by IFA staining methods, results were negative in all tests that included either conjugate.

DISCUSSION

White-footed mice can carry antibodies to multiple tick-borne pathogens in Connecticut. These results parallel those reported for human sera (Magnarelli et al., 1995a). Antibody test results for human sera and clinical findings do not exclude that HME is probably present along with HGE in some tick-infested areas of southern New England (Rynkiewicz and Liu, 1994; Hardalo et al., 1995; J. S. Dumler, unpubl.). That rodents are infected with *B. burgdorferi* and *B. microti* in northeastern United States is well recognized (J. F. Anderson et al., 1986, 1991). Coexistent infections of these pathogens in humans (Marcus et al., 1985) and simultaneous transmission of these agents by ticks (Piesman et al., 1987) also are apparent. However, serologic evidence of *E. chaffeensis*, *E. equi*, or both infections in humans and mice is evidence that other pathogens occur in forested settings. Our finding antibodies to different species of ehrlichiae in white-footed mice shows that these rodents are naturally exposed to these or closely related pathogens. Detection of DNA of the HGE agent (*E. equi* or a closely related organism) in *I. scapularis* (Magnarelli et al., 1995b; Pancholi et al., 1995; Telford et al., 1996), a tick that frequently parasitizes white-footed mice, is further evidence for a possible enzootic cycle that includes these mammals.

On the basis of tick bite records of persons who had HGE (Chen et al., 1994), *I. scapularis* and *Dermacentor variabilis* (American dog tick) are suspected vectors. Although the DNA of *E. chaffeensis* was not found in *I. scapularis* in Connecticut (Magnarelli et al., 1995b), further studies are needed to verify whether this tick species and *D. variabilis* are vectors of this agent or the HGE organism. Under experimental conditions, *Amblyomma americanum* (lone-star tick) transmitted *E. chaffeensis* and a granulocytic form, *Ehrlichia ewingii* (tribe *Ehrlichieae*) to dogs (Anziani et al., 1990), and passed *E. chaffeensis* to white-tailed deer (Ewing et al., 1995). Therefore, it appears that multiple tick species may be transmitting different ehrlichiae to mammals in widely separated sites.

The relatively high prevalence of multiple infections in white-footed mice is probably epizootiologically important. Granted, even though some mice were shown in isolation studies to be co-infected with *B. burgdorferi* and *B. microti*, presence of antibodies to *E. chaffeensis* and *E. equi* do not necessarily mean that there were concurrent infections with one or both of these ehrlichiae. Nonetheless, simultaneous borrelial, babesial, and ehrlichial infections may occur in some instances. The pathogenicity of ehrlichiae in mice, influence of these etiologic agents on the host's immune system, and relevance of multiple infections to reservoir competency for one or more human pathogens need further investigation.

Interpretation of antibody test results without such supportive laboratory findings as culture work or PCR analyses can be difficult. Based on our tests, antibodies to *E. chaffeensis*, *E. equi* or both occurred in mice. Seven mouse sera contained antibodies to both *E. chaffeensis* and *E. equi*. There did not appear to be interspecies cross-reactivity problems with *B. burgdorferi* and *B. microti*. On the basis of specificity studies with human sera, there was little or no serologic cross-reactivity when homologous antibodies to these ehrlichiae or to *B. burgdorferi* and *B. microti* were tested with heterologous antigens (Dumler et al., 1995; Magnarelli et al., 1995a,b). There is notable divergence between *E. equi* and *E. chaffeensis* when results of

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analyses of 16S rRNA gene sequences are compared (Chen et al., 1994). Similarly, *B. burgdorferi*, *B. microti*, and ehrlichiae are phylogenetically unrelated. We recognize, however, that some serologic cross reactivity may still occur between *E. chaffeensis* and *E. equi*, particularly if antibody titers to one or both pathogens are high. Also, based on PCR analyses and DNA detection methods, a unique *Ehrlichia*-like agent is believed to be present in white-tailed deer in Oklahoma and Georgia (USA) (Dawson et al., 1996). Therefore, these or other undescribed ehrlichiae may be present in white-footed mice or *I. scapularis* ticks and may be closely related to the ehrlichial species used as antigens in our antibody analyses. Clearly, additional work is needed to isolate and identify ehrlichiae that infect mice and ticks in areas where HGE and HME are reported. Progress has been made in isolating ehrlichiae in tick and mammalian cell cultures (Goodman et al., 1996; Munderloh et al., 1996). Moreover, PCR and DNA detection methods, which show promise in the diagnosis of HGE and HME infections (Chen et al., 1994), should be further evaluated with challenged mice to determine the reliability of detecting DNA of various ehrlichiae in blood or other tissues.

Indirect FA staining methods, including either conjugated antibody, were suitable for detecting antibodies to *E. chaffeensis* and *E. equi*. However, these analyses were time-consuming and, when testing with *E. equi* antigen, slide preparations were sometimes difficult to read because the number of infected equid neutrophils fixed to slides varied. More refined, automated antibody tests are needed. Use of an ELISA with purified recombinant antigens, such as outer surface protein C, is suitable for diagnostic testing of early human Lyme disease (Padula et al., 1994). An ELISA with specific ehrlichial antigens could likewise be used to quantitate concentrations of antibodies to *E. chaffeensis* and *E. equi*. For example, in Western blot analyses with sera from human patients convalescing from HGE, from horses and dogs infected with *E. equi*, and from cattle with *Ehrlichia phagocytophila* infection, antibodies have been detected to a single dominant and highly specific antigen of *E. equi* having a molecular mass of 44 kDa (Dumler et al., 1995). Similar testing by immunoblotting procedures is required to determine if laboratory-infected mice frequently produce antibodies to this or other key polypeptides. We propose that laboratory-born white-footed mice or another suitable rodent host be challenged and used as a model to monitor a variety of ehrlichial infections, characterize immune responses, and to further evaluate antibody or DNA detection procedures. Results of these studies will be needed to assess reservoir competency of rodents when feeding experiments with ticks are conducted and to aid in the development of improved diagnostic assays for human and veterinary ehrlichial infections.

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